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New approaches for the identification of antivirulence agents based on LsrK inhibition:

from assay development to screening campaigns

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DOCTORAL DISSERTATION

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To Gino, my grandfather.
you have always believed in me
you have always been with me
and today, wherever you are, you are smiling.

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Abstract

The failure of current antibiotics to address the spread of bacterial resistance has highlighted the need of new antimicrobial agents which would apply less selective pressure. To answer that, great interest has arisen towards antivirulence agents, compounds which target virulence factors and thus bacterial pathogenicity. As no link occurs between growth and virulence, antivirulence agents are considered less prone to promote resistance development.

Due to its role as regulator in the establishment of infections, quorum sensing (QS), a communication strategy among bacteria mediated by the trafficking of small molecules called autoinducers (AI), has been widely investigated for the development of QS inhibitors with the aim of limiting bacterial virulence.

This study aimed to develop new assays for the discovery of inhibitors targeting LsrK, a key kinase for autoinducer 2 (AI-2) mediated QS establishment in enteric bacteria. LsrK in fact phosphorylates the AI-2 which, only in the phosphorylated form, can bind to the LsrR repressor and enhance the response to QS signals via activation of the *lsr* operon. A target-based assay based on the detection of remaining ATP after the LsrK-catalyzed reaction, was developed and utilized for screening of three different compound libraries. The libraries were chosen according to different criteria to increase the probability of finding LsrK inhibitors. Specifically in study I, the MicroSource Spectrum library was chosen due to its chemical diversity, in study II a collection of compounds was selected by virtual screening using a 3D LsrK model and in study III a small set of DPD analogues designed to target the LsrK substrate binding pocket was screened. The best hits from the three campaigns were harpagoside and rosolic acid, discovered by screening the MicroSource library. These compounds were also active in cell-based AI-2 mediated QS assay. However, the positive hits derived by study II and III provided interesting information about the catalytic site of LsrK. SAR studies in fact demonstrated that the best hits interact with Tyr 285 which may thus play a key role in the enzymatic activity.

To facilitate the confirmation of hits selected by target-based assay and to offer a new tool for the rapid identification of QS inhibitors, in study IV a new bioreporter strain was created. This bioreporter emits luminescence as

response to AI-2 mediated QS activation. The assay has an easy set-up, requires only addition steps and is thus easily automatable. Additionally, the use of a mutant strain with reduced efflux activity allows enhanced intracellular accumulation of tested compounds increasing the chance to select active molecules to be used as scaffold for further development. The assay was used to test a set of 91 compounds selected to target the ATP binding site of LsrK. The same set of compounds was also tested in the target-based LsrK inhibition assay and the results were merged to lead to a final list of 24 potential QS inhibitors. Among those, 6 showed activity also against LsrK, which suggests that they decrease response to QS by inhibiting the kinase whereas 18 showed activity only on the cell-based assay implying that they target other components of the pathway. Further investigations are needed to define their mode of action.

The inhibitors we found represent the first LsrK inhibitors and although their IC_{50} values limit follow-up studies and clinical applications, they increased our knowledge on LsrK and are useful scaffolds to better understand the enzyme-substrate interactions and to design compounds with improved properties. Furthermore, the AI-2 mediated QS interference assay represents an additional tool for the identification of QS inhibitors as it not only complements target-based assay but also allows to find new classes of QS inhibitors which act on the *lsr* pathway.

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List of original publications

The thesis is based on the following original publications which are referred in the text by their roman numbers (I-IV):

I. Gatta V, Ilina P, Porter A, McElroy S, Tammela P; Targeting Quorum Sensing: High-Throughput Screening to Identify Novel LsrK Inhibitors. *Int J Mol Sci* **2019**; 20(12). pii: E3112.

II. Medarametla P[†], Gatta V[†], Kajander T, Laitinen T, Tammela P, Poso A; Structure-Based Virtual Screening of LsrK Kinase Inhibitors to Target Quorum Sensing. *ChemMedChem* **2018**; 13(22):2400-2407.

[†] These authors contributed equally to the work

III. Stotani S, Gatta V, Medarametla P, Padmanaban M, Karawajczyk A, Giordanetto F, Tammela P, Laitinen T, Poso A, Tzalis D, Collina S; DPD-Inspired Discovery of Novel LsrK Kinase Inhibitors: An Opportunity To Fight Antimicrobial Resistance. *J Med Chem* **2019**; 62(5):2720-2737.

IV. Gatta V, Tomašič T, Ilaš J, Zidar N, Mašič LP, Barančoková M, Frlan R, Anderluh M, Kikelj D, Tammela P; A new cell-based AI-2-mediated quorum sensing interference assay in screening of LsrK-targeted inhibitors. *Chembiochem* **2020**; [Epub ahead of print]

Author´s contributions

I. Viviana Gatta participated to the design of the study and executed the experimental work. She analyzed the data and wrote the manuscript in collaboration with the co-authors.

II. Viviana Gatta participated in the design of the study and was responsible for the execution and data analysis of the biological studies. She collaborated with the co-authors to write and revise the manuscript.

III. Viviana Gatta contributed to the design of the study and participated to the experimental work. She executed the biological experiments, took part in the interpretation of the results and in the writing of the manuscript.

IV. Viviana Gatta participated in the design of the study. She executed the experiments, interpreted the results and wrote the manuscript in collaboration with the co-authors.

Abbreviations

ADP adenosine diphosphate

AHL acyl homoserine lactone

AI autoinducer

AI-2 autoinducer-2

AI-3 autoinducer-3

AIP autoinducing peptide

AMP antimicrobial peptide

ATCC American Type Culture Collection

ATP adenosine triphosphate

BSA bovine serum albumin

cIAIs Complicated intra-abdominal infections

cUTIs Complicated urinary tract infections

DMSO dimethyl sulfoxide

DSF *cis*-11-methyl-2-dodecenoic acid

DPD (*S*)-4,5-dihydroxy-2,3-pentanedione

ECM extracellular matrix

EMA European Medicines Agency

EPS exopolysaccharides

ESBLs extended-spectrum β-lactamases

ESKAPE *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.

FDA Food and Drug Administration

FIMM Institute for Molecular Medicine Finland

FRET fluorescence resonance energy transfer

HAV hospital acquired pneumoniae

HHQ 2-heptyl-4-hydroxyquinoline

HTS high throughput screening

IPTG isopropyl β -D-1-thiogalactopyranoside

LB lysogenic broth

mAB monoclonal antibody

MDROs multidrug resistant organisms

MRSA methicillin resistant *S. aureus*

MTAN methylthioadenosine/S-adenosylhomocysteine nucleosidase

NIG National Institute of Genetic

ONPG 2-nitrophenyl β-D-galactopyranoside

PAβN phe-arg-β-naphtylamide dihydrochloride

PCR polymerase chain reaction

PDR pan-resistant organisms

PQI 2-heptyl-3-hydroxy-4(1H)-quinolone

QS quorum sensing

PTS phosphoenolpyruvate phosphotransferase system

RT room temperature

TEA triethanolamine

TLC thin layer chromatography

UPEC uropathogenic *E. coli*

VAP ventilated acquired pneumoniae

XDR extensively resistant organisms

WHO World Health Organization

1. Introduction

Antibiotics are considered as one of the most significant innovations in the history of medicine as they have improved and saved millions of lives throughout the years.

The research for compounds with antibacterial properties dates back to the early 1900s, when, with the discovery of the first anti-syphilis drug, Paul Ehrlich marked the beginning of the "modern era" of antibiotics. Ehrlich's approach included the systematic screening of hundreds of synthetic compounds on syphilis affected rabbits and it became an important milestone for drug discovery in pharmaceutical research [1].

Subsequently, with the discovery of penicillin by Fleming, the interest towards synthetic chemistry decreased in favor of natural products which were exploited as preferential source of effective and chemically diverse compounds. Most of the classes of antibiotics actually in use were discovered and characterized in those days, between the 1940s and 1960s, which are known as "golden era" of antibiotics. Only in the 1970s there was a return to synthetic approach to further optimize the compounds already available [1] and no new class of antibiotics was introduced till 2000 with the discovery of linezolid [2].

The lack of advancement in antibiotic discovery together with the misuse of antibiotics, with self-prescription and with the natural genetic variability of bacteria, has significantly reduced the effectiveness of available treatments [3, 4]. The term multidrug resistant organism (MDR) has been introduced to categorize bacteria which are resistant to many of the current antibiotics and becoming a threat to human health [5]. Among those, *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp., better known as the "ESKAPE" panel from their initials, are particularly relevant as they are considered critical threats to human life thanks to their ability to escape the action of several antibiotics [5, 6, 7].

According to the World Health Organization (WHO), humanity is approaching a post-antibiotic phase when common infections will be deadly

for millions of people [8]. Although it may sound excessively alarmistic, this perspective is highlighting the need of a common effort to discover new antibiotics together with more sophisticated diagnosis methods, smarter delivery systems and new approaches to limit and prevent the spread of resistance.

Aiming to remark the importance of a multidisciplinary and innovative approach to fight bacterial infections, the following review provides an overview on alternative strategies which are currently been investigated to control bacterial infections.

2. Review of literature

2.1. Resistance to antibiotics

Traditional antibiotics target vital bacterial processes causing bacterial death (bactericidal effect) or preventing bacterial growth (bacteriostatic effect). Interfering with life functions has posed a strong selective pressure which, coupled with the spontaneous tendency of bacteria to mutate, has caused the spread of several resistant strains which are not susceptible to the currently available treatments.

Resistance can be defined as the ability of bacteria to grow in the presence of normally cytotoxic concentration of antibiotic. Consequently, the resistant strain will get competitive advantage over the other cells becoming the dominant population. Bacteria can acquire resistance through mutations or transfer of genetic material which prevent the interaction of the antibiotic with its target. Additionally, chemical modifications can occur on the antibiotics, leading to the loss of the active motif. The most famous example is the hydrolysis of β-lactam ring catalyzed by β-lactamases which causes the degradation of penicillins, cephalosporine and carbapemenes [9]. The development of analogues of β-lactams in the following years have been coupled with the evolution of extended-spectrum β-lactamases (ESBls) and carbapemenases which have been transferred among bacteria via horizontal gene transfer with serious consequences for the treatment of infections. Other known chemical modifications include the acetylation, adenylation or

phosphorylation of the hydroxyl and amino-group in aminoglycosides with decrease of bacterial susceptibility to this class of antibiotics [10, 11].

Target modifications may reduce the accessibility of the target or modify its structures. Binding of aminoglycosides to their ribosomal targets can be prevented by RNA methylation. Clinically relevant strains have also developed proteins which compete with antibiotics for the binding to the target [12]. Tet proteins bind to ribosomes and displace tetracycline by mimicking the activity of elongation factors during protein synthesis [13, 14]. The quinolone resistance protein Qnr can mimic DNA and compete with fluoroquinolones for the binding to DNA gyrase and topoisomerase IV [15]. Although the mechanism is more complex as it affects enzymes that are vital for bacteria, mutations in the DNA gyrase and topoisomerase IV can also occur and contribute to resistance to fluoroquinolones by altering the binding pocket. Mutations in the gene encoding for the RNA polymerase cause amino acidic substitutions which prevents the binding of rifamycin whereas the regular enzymatic activity is conserved [16].

In addition to acquired resistance, bacteria can also be intrinsically resistant to antibiotics i.e. resistance is due to structural characteristics. The outer membrane of Gram-negative bacteria is an excellent resistance mechanism as it makes them resistant to several antibiotics that are active against Gram-positives and it also represents the main obstacle for the discovery of active molecules [17]. By regulating the expression of channels on the membrane, bacteria can also modulate their permeability. The downregulation of porins, is very often coupled with the overexpression of efflux pumps which actively expel xenobiotics out of the cell [18]. Mutations on the main regulators of the efflux pump system may affect the expression and the activity of the pumps. The exchange of genetic material encoding for the efflux pumps among bacteria provides them with a significantly complex and diverse efflux pump network [19,20,21,22].

The coexistence and synergism of the various resistance mechanisms led to the evolution of strains which are classified as MDR, XDR and PDR, according to the number of antimicrobials they can escape. MDR are strains resistant to at least 1 antibiotic from 3 or more antimicrobial classes whereas bacteria resistant to all the available treatments are defined pan-resistant organisms (PDR). An intermediate category has also been introduced to classify those organisms which are only susceptible to one or two categories

of antibiotics. Those are defined as extremely or extensively drug resistant bacteria (XDR) [23].

2.2. MDR and XDR pathogens: current treatments and new perspectives

Few options are currently available for the treatment of MDR and XDR Gram-negatives which are one of the principal causes of death among hospitalized but also healthy individuals [24]. Among those colistin and fosfomycin are considered as the last frontier treatments when no other options are available. Colistin is a cyclic heptapeptide linked to a fatty acid chain produced by *Bacillus polymyxa var. colistinus* and active against aerobic Gram-negative bacteria [25, 26] It promotes cell damage by altering bacterial cell membrane permeability after binding to lipid A, a bacterial membrane component [27]. Due to the required high dosages and the high frequency of resistance and mutants, the use of monotherapy is controversial and the simultaneous administration of colistin with other antimicrobial agents is preferred to increase effectiveness and reduce the side effects [25, 28]. Combinations of colistin with carbapenem, fosfomycin, rifampin have shown improved efficacy for the treatment of problematic strains compared to treatment with single compounds [29, 30, 31]. Emergence of mutants is also generally less frequent. For colistin-resistant strains, fosfomycin, a bactericidal agent produced by several strains of *Streptomyces*, can be administrated [32]. Fosfomycin inhibits peptidoglycan synthesis and thus prevents the formation of bacterial cell wall with consequent cell damage [33]. It shows good penetration into tissues associated with low side effects [34]. However, combination therapy is advised to reduce resistance occurrence although an ideal drug to be co-administrated with fosfomycin has not yet been identified [35, 36, 37].

Ultimately, aminoglycosides have also been used for the treatment of XDR, especially in combination with other classes of antibiotics with promising results [38, 39]. Limitation is represented by different susceptibility of bacteria due to the expression of different classes of aminoglycosides modifying enzymes and also the need of surveillance for the administrated doses in order to avoid toxicity [40].

In the current scenario of available antibiotics, the key element seems to be combination as the simultaneous administration of a known antibiotics with a new agent restoring bacterial susceptibility has been proven to be highly effective against critical strains.

As proof of concept for the effectiveness of combination therapy for the treatment of MDR and XDR, among the few new treatments recently approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) (Table 1), three of them are combinations of a traditionally used antibiotic complemented by an ESBLs inhibitor. The activity of ceftazidime, ceftolozone and meropenem is in fact restored in presence of avibactam, tazobactam and vaborbactam, respectively [41, 42, 43].

Additionally, plazomycin, a new generation aminoglycoside, was approved for the treatment of *Enterobacteriaceae*. The ability to enter lungs makes it also a candidate for the treatment of ventilated associated pneumoniae (VAP) in combination with β-lactamase inhibitors. Eravacycline is a synthetic fluorocycline active against Gram-negative *bacilli* and Gram-positive *cocci* with limited side effects.

Table 1: List of approved drugs for the treatment of Gram-negative infections between 2014 and 2019 [44]

Drug	Class	Status
Ceftazidime/avibactam	combination of avibactam, a β-lactamase inhibitor, with ceftazidime	- approved by FDA and EMA for the treatment of cUTI[1], cIAI[2], HAP[3], and VAP[4] - approved by EMA for the treatment of infections by aerobic Gram-negative when limited options are available
Ceftolozane/ Tazobactam	combination of tazobactam, a β-lactamase inhibitor, with ceftolozane	- approved by FDA and EMA for the treatment of cIAI (in combination with metronidazole) and cUTI including AP[5]
Meropenem/ Vaborbactam	combination of vaborbactam, a β-lactamase inhibitor, with meropenem	- approved by FDA for the treatment of cUTI, including AP - approved by EMA for the treatment of cUTI, cIAI, VAP, HAP, and infections due to aerobic

[1] Complicated urinary tract infections
[2] Complicated intra-abdominal infections
[3] Hospital acquired pneumonia
[4] Ventilated associated pneumonia
[5] Acute pyelonephritis

		Gram-negatives when limited treatment options are available
Plazomycin	semisynthetic aminoglycoside	- approved by FDA for the treatment of cUTI, including AP
Eravacycline	synthetic fluorocycline	- approved by FDA and EMA for the treatment of complicated intra-abdominal infections

The above options are still meant to be used in combination with colistin or fosfomycin for the treatment of severe infections. In particular ceftazimide/avibactam and meropenem/vaborbactam are indicated for the treatment of CRE infections whereas cefozolane/tazobactam is indicated for the treatment of MDR *P. aeruginosa*.

2.3. Antivirulence strategies

Colistin, fosfomycin and the new combination therapies, despite their potential for the treatment of XDR and MDR, cannot fulfill the need of new antibiotics, especially when considering that they are bactericidal and, consequently, will apply a strong pressure for the selection of mutants. Surveillance and consciousness in the use of those antibiotics is thus fundamental to save more human lives, offering at the same time a time window to scientists for the discovery of alternative approaches which may reduce the selective pressure. As big pharma companies exited the field of antimicrobials due to low profit margins, several institutions, including small companies and academic groups, have in fact contributed to the development of new antimicrobial strategies which may translate into clinic in the coming years. According to a recent review published in Nature Review Microbiology, 407 projects are on-going to discover new treatments against bacteria. Among those, 46% still focuses on compounds with antibacterial properties, including either derivatives of traditional antibiotics or new targets, whereas the 56% is looking at new strategies spanning from phage therapy to antibodies and vaccines (Figure 1) [45].

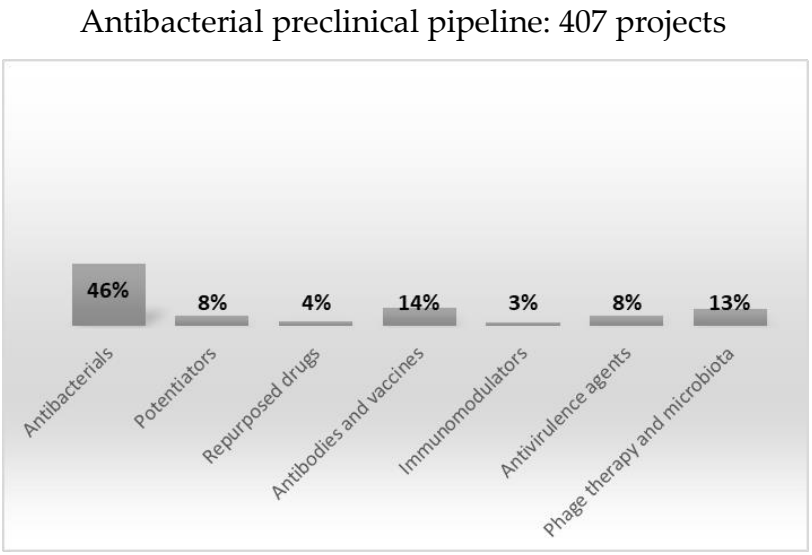


Figure 1: Areas of research for new antimicrobials [45].

A portion of new innovations is reserved to the development of antivirulence agents which target virulence factors, responsible for bacterial pathogenicity. This approach aims to make bacteria inoffensive so that they can be naturally cleared by the host immune system [46, 47, 48]. The advantage of antivirulence therapy is primarily the low selective pressure. In fact, since antivirulence agents do not have an impact on growth, the treatment is considered less prone to promote resistance.

A second benefit is specificity. Many virulence factors are species specific or limited to a restricted number of strains, and thus do not impair endogenous bacteria which normally do not possess virulence factors. Additionally, specificity also lowers the risk of resistance as the horizontal transfer of mutated genes is unlikely to happen [49, 50]. On the other hand, it limits the applicability of antivirulence drugs quite significantly as each patient may carry a different strain with different mutations so that data derived from clinical trials and surveillance studies are hard to interpret due to the high variability. The lack of an agreement about final read-outs to monitor effects also impacts the advancement of antivirulence drugs towards clinical trials. In fact, whereas for traditional antibiotics a therapy is successful when the

bacterial cells are dead, for antivirulence agents cell death is an undesired effect and multiple parameters should be analyzed such as reduced gravity of the infection or level of the immune response [51].

However, the interest of the scientific community towards antivirulence agents is considerably high. The most interesting aspect of antivirulence therapies is the number and variety of targets. In fact, host colonization, motility, toxin production and release, and biofilm formation are all contributing to virulence and each of them is a complex process which involves several proteins, potentially suitable as targets to disrupt pathogenicity.

To initiate the infection, pathogens need to adhere and colonize the host tissue. Bacterial adhesion apparatus generally includes a protruding structure associated with adhesins, proteins responsible for the recognition and binding to the host tissue. FimH mediates the adhesion of uropathogenic *E. coli* (UPEC) to the uroepithelium by binding to mannosylated components. Thus mannosides have the potential to prevent UPEC adhesion to the bladder cells by binding FimH [52]. UPEC also colonizes kidneys by using Fim1H which binds to β-2-galactoside. N-acetyl galactosamine inhibits the binding and ensures full recovery of infected mice when administrated in combination with mannosides [53]. *Enterobacteriaceae* can translocate the intimin receptor Tir to the host cell via the type III translocation system and interact with it by intimin protein forming stable interactions which support colonization and damage [54]. The treatment with peptides which interfere with the correct transporter assembly have successfully prevented lesions formation in murine model [55].

Toxins are small proteins secreted by bacteria to damage the host and impair its metabolic pathways [56]. The functionality of toxins requires the interaction with a host receptor activating lethal pathways for the cells. *S. aureus* produces a variety of toxins which are responsible for its pathogenicity. α-hemolysin kills blood and immune cells by disrupting their cellular membranes [57]. Treatment with monoclonal antibodies significantly reduces the effect of toxins and cures the infections. MED14893 mAB inhibits α-hemolysin oligomerization and its interaction with the receptor reducing bacteremia and lesions due to *S. aureus* in mice [58]. A mice model of lung co-infection of *S. aureus* and Gram-negative bacteria regained health after preimmunization with an antitoxinA mAB [59]. Liposomes containing high percentage of cholesterol and sphingomyelin

were able to capture toxins based on their affinity for cholesterol with a positive effect on animals infected with *S. pneumoniae* and *S. aureus* [60]. TcdA and TcdB toxins, from *C. difficile*, cause cell damage and spreading of bacteria by interacting with the Ras superfamily of small GTPases. Different conformations of TcdB have been isolated and correspond to different level of virulence [61]. The sequence of the high virulence variant of TcdB was used to design peptide which could destabilize the protein and reduce its activity [62].

The type III translocation system is used by Gram-negative bacteria to inject toxins into the host cell. Several compounds have been proposed to prevent the assembly of the transporter and mAB targeting the PcrV, protein associated to the needle, have entered clinical trials for the treatment of *P. aeruginosa* infections [63].

Despite identifying toxins and clarifying their mechanism of action may be difficult and dispendious, anti-toxin treatments were the first antivirulence agents approved by FDA but many others are under investigations (Table 2). BabyBIG and BAT were approved in 2003 and 2013, respectively, for the treatment of *C. botulinum* infections [64, 65]. *C. botulinum* secretes BoNT, a neurotoxin causing paralysis by impairing neuromuscular junctions. BabyBIG is an immunoglobulin derived by human donors immunized with BoNT (type A-E) used for the treatment of infant botulism whereas BAT derives from immunization of horses and has a wider spectrum targeting also BoNT type H. Raxibacumab [66] and obiltoxaximab [67] were approved for the treatment of *B. antracis* infections. Both mAbs target the protective antigen (PA) and prevent the PA mediated-internalization of the lethal factor and oedema factor, toxins causing the anthrax infections. Bezlotoxumab, approved in 2016 for the treatment of *C. difficile* infections [68], targets TcdB, an exotoxin which, together with TcdA, causes *C. difficile* virulence by damaging enterocytes.

Table 2: Antivirulence agents at different stages of drug development in 2017 [69]

a) Approved drugs

Compounds		Organisms	Molecular target(s)	Status
BabyBIG	Human, mostly IgG, plasma-derived immune globulin	*Clostridium botulinum*	BoNT serotypes A and B	FDA approved
BAT	Equine, Fab[6] and $F(ab')_2$; plasma-derived immunoglobulin	*Clostridium botulinum*	BoNT serotypes A–G	FDA approved
Raxibacumab	Human, mAb[7] IgG1	*Bacillus anthracis*	Protective antigen of anthrax toxin	FDA approved
Obiltoxaximab	Human, mAb IgG1	*Bacillus anthracis*	Protective antigen of anthrax toxin	FDA approved
Bezlotoxumab (also known as MDX-1388)	Human, mAb IgG	*Clostridium difficile*	TcdB	FDA approved

b) Potential drugs in clinical trials

Compounds		Organisms	Molecular target(s)	Status
MEDI4893	Human, mAb IgG1	*Staphylococcus aureus*	α-Toxin	Phase II
AR-301	Human, mAb IgG1	*Staphylococcus aureus*	α-Toxin	Phase II
ASN-100 (a combination of ASN-1 and ASN-2)	Human, mAb IgG1	*Staphylococcus aureus*	α-Toxin, PVL, LukED, LukGH and γ-haemolysin	Phase II
MEDI3902 (also known as BiS4αPa)	Engineered bispecific antibody	*Pseudomonas aeruginosa*	Psl and PcrV	Phase I and II
Shigamab (also known as cαStx2)	Human–mouse chimeric mAb	Stx-producing *Escherichia coli*	Stx2	Phase I

[6] Fragment antigen-binding
[7] Monoclonal antibody

c) Potential drugs in preclinical studies

Compounds		Organisms	Molecular target(s)	Status
11D and 4C	Chimpanzee, mAbs IgG1 and IgG3	*Bacillus anthracis*	PGA	Preclinical (including animal models)
F26G3, F24F2 and F26G4	Mouse, mAb IgG3	*Bacillus anthracis*	PGA	Preclinical (including animal models)
Ebselen	Organoselenium	*Clostridium difficile*	TcdA and TcdB	Preclinical (including animal models)
6e	3,6-Disubstituted triazolothiadiazole compounds	*Staphylococcus aureus*	SrtA	Preclinical (including animal models)
Savirin	3-(4-Propan-2-ylphenyl) sulfonyl-1H-triazolo [1,5-a] quinazolin-5-one	*Staphylococcus aureus*	AgrA	Preclinical (including animal models)
RS2-1G9	Mouse, mAb IgG	*Pseudomonas aeruginosa*	3-Oxo-C12-AHL	Pre-clinical
INP1855	Hydroxyquinoline	*Pseudomonas aeruginosa*	Possibly the ATPase of T3SS (PscN) and flagellum	Preclinical (including animal models)
M64	Benzamide-benzimidazole	*Pseudomonas aeruginosa*	MvfR	Preclinical (including animal models)
Compounds 37a and 37b	Phenoxyacetamide	*Pseudomonas aeruginosa*	PscF	Preclinical
E22	Phenoxyacetyl homoserine lactone (AHL mimic)	*Pseudomonas aeruginosa*	RhlR	Preclinical
mBTL	Halogenated thiolactone	*Pseudomonas aeruginosa*	RhlR	Preclinical (showed efficacy in a *Caenorhabditis elegans* model)
itc-12 and itc13	Isothiocyanate-AHL	*Pseudomonas aeruginosa*	LasR	Preclinical
C30	Halogenated furanone	*Pseudomonas aeruginosa*	LasR	Preclinical (including animal models)
3-Oxo-C12-	Acyl-	*Pseudomonas*	LasR	Preclinical

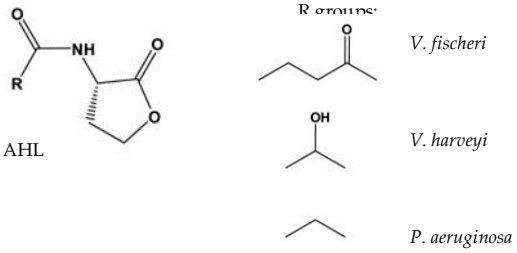
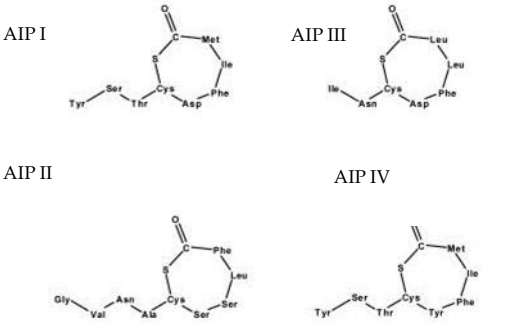
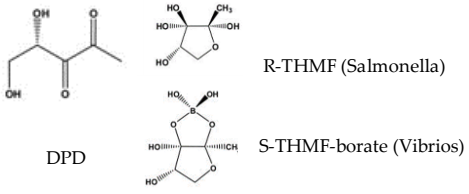
(2-amino-cyclohexanone)	aminocyclohexanone (AHL mimic)	*aeruginosa*		
mAb926	Mouse, mAb IgG	Uropathogenic *Escherichia coli*	FimK	Preclinical (including animal models)
Compound 22	Biarylmannoside	Uropathogenic *Escherichia coli*	FimK	Preclinical (including animal models)
ec240	Ring-fused 2-pyridones	Uropathogenic *Escherichia coli*	Unknown, but decreases piliation	Preclinical
KP3	Engineered, scFv-Fc antibody	*Klebsiella pneumoniae*	MrkA	Preclinical (including animal models)
LpxC-1	Methylsulfone hydroxamate	*Acinetobacter baumannii*	LpxC	Preclinical (including animal models)
Deferiprone	3-Hydroxy-1,2-dimethylpyridin-4(1H)-one	*Acinetobacter baumannii*	Ferric iron	Preclinical
Virstatin	4-[N-(1,8-naphthalimide)]-n-butyric acid	*Acinetobacter baumannii*	Unknown, but decreases piliation	Preclinical
ZBzl-YAA5911	Cyclic peptide	*Enterococcus faecalis*	FsrC	Preclinical (including animal models)
Anti-EbpA immune sera	Mouse, immune sera	*Enterococcus faecalis*	EbpA	Preclinical (including animal models)
Stx1e-1, Stx1e-2, Stx1e-3 and Stx1e-4	Mouse, mAbs IgG1 and IgG2a	*Enterobacter spp.*	Stx1e	Preclinical
SMITB14	Mouse, mAb IgG1	*Mycobacterium tuberculosis*	Lipoarabinomannan	Preclinical (including animal models)
2E9IgA1	Human, mAb IgA1	*Mycobacterium tuberculosis*	α-Crystallin	Preclinical (including animal models)
4057	Mouse, mAb IgG3	*Mycobacterium tuberculosis*	Heparin-binding haemagglutinin	Preclinical (including animal models)

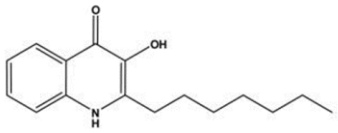
2.3.2. Quorum sensing inhibition

As virulence is the result of the synergism of multiple virulence factors, disrupting virulence regulatory system may offer a unique opportunity to disarm bacteria by targeting several virulence factors simultaneously. For this purpose, quorum sensing (QS) has raised a great interest in the past years. In fact, this network, which relies on release and recognition of small molecules called autoinducers (AI), has been proposed as regulator of several bacterial group behaviors such as host colonization and pathogenicity [70, 71].

The discovery that bacteria can communicate dates to the 70s when LuxI and LuxR were proven to be regulators of luminescence in *V. fischeri* [72, 73]. Later homologous genes were identified in other species together with numerous autoinducer classes (Table 3).

Table 3: Examples of autoinducers and their spectrum of activity {74}

Autoinducer class	Spectrum	Structures:
Acyl homoserine lactone (AHL)	Gram-negatives	
Oligopeptides	Gram-positives	
Autoinducer-2	Gram-negatives/ Gram-positives	

PQS/IQS	*Pseudomonas aeruginosa*	 PQS

Acyl homoserine lactone (AHL), the most known autoinducer in Gram-negatives, is characterized by a lactone ring connected to an acyl chain with various length, oxidation and saturation. Synthases from the Lux1 family catalyze AHL synthesis starting from the S-adenosylmethionine [75]. The signal moves to the extracellular environment where, after exceeding a certain threshold, binds to LuxR receptor with an impact on gene expression.

Gram-positive bacteria secrete oligopeptides as QS mediators. Oligopeptides are released into the extracellular environment where they undergo structural modification before interacting with the cognate receptor on bacterial membrane [76]. The binding initiates a phosphorylation cascade which ends with the de-repression of QS responsive genes. In *S. aureus* the autoinducing peptide (AIP) controls the accessory gene regulator system (*agr*). The *agr* system contains two gene clusters: RNAII and RNAIII. RNAIII includes QS responsive genes whereas RNAII encodes for AgrB, AgrC, AgrD and AgrA, involved in production and processing of the AIP. AIP derives from AgrD, a precursor peptide which is processed by AgrB, a transmembrane peptidase, and SspB, a type 1 signal peptidase. Once it is released extracellularly, AIP binds to the receptor AgrC starting a phosphorylation cascade which ends with the binding of the phosphorylated AgrA to the P2 and P3, two promoters which regulate the transcription of RNAII and RNAIII (Figure 2)[77].

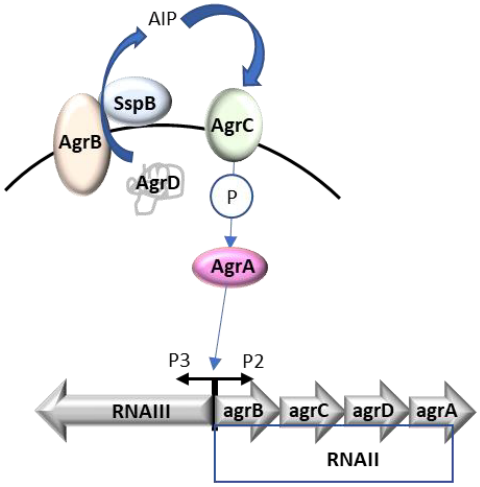


Figure 2: Schematization of *agr* mediated QS system in *S. aureus.*

AI-2 is used as QS mediator by both classes of bacteria for interspecies and intraspecies communication. Methylthioadenosine/*S*-adenosylhomocysteine nucleosidase (MTAN) and the metalloenzyme LuxS synthetize 4,5-dihydroxy-2,3-pentanedione (DPD), a highly unstable molecule which exists as balance of several cyclic forms [78]. Whereas AI-2 synthetic pathway seems to be highly conserved, AI-2 processing may vary considerably. As general mechanism AI-2 is extruded and binds to a receptor after a certain concentration is reached. According to current knowledge, two classes of receptors have been well described. In *V. harveyi*, AI-2 interacts with LuxP, a periplasmatic protein which modulates the activity of the kinase LuxQ. At low cell density the complex LuxPQ phosphorylates the downstream proteins LuxU and LuxO which, in the phosphorylated form, promote the transcription of regulatory sRNAs, named Qrr, with the consequent inhibition of LuxR, a regulator of high cellular density behavior [79, 80]. When the furanosyl borate diester form of AI-2 binds to the receptor, the complex acts as phosphatase with consequent dephosphorylation of LuxO, inhibition of the sRNAs transcription and LuxR expression and activity (Figure 3) [81].

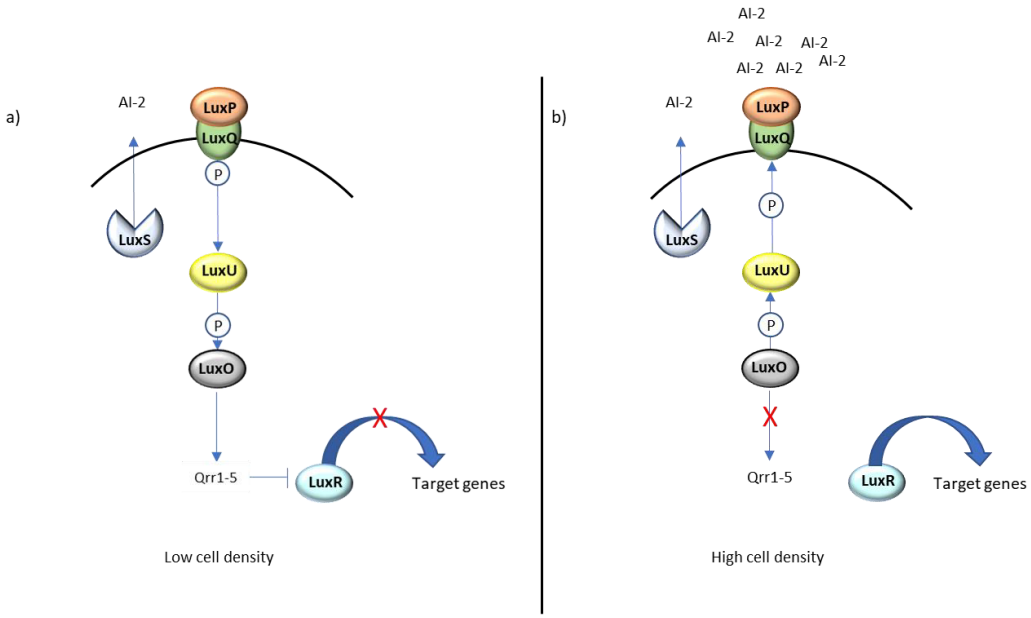


Figure 3: Schematization of AI-2 mediated QS in *V. harveyi:* a) at low cell-density; b) at high cell-density.

In enteric bacteria, LsrB-like receptors recognize the signal which is internalized by a transporter formed by two transmembrane proteins, LsrC and LsrD, linked to a third protein, LsrA, with ATPase activity. The receptor and the transporter's components are encoded by *lsrACDBFGE* operon which also encodes for LsrG and LsrE, responsible for AI-2 degradation. LsrK, and LsrR, which respectively encode for a kinase and a repressor, are located adjacently to the operon and are transcribed divergently. Inside the cell, LsrK phosphorylates the signal molecule which only in the phosphorylated form can bind and inactivate AI-2 repressor LsrR, enhancing the transcription of the *lsr* operon and promoting the response to QS [82]. The LsrB-like receptor mediated pathway is under control of the phosphoenolpyruvate phosphotransferase system (PTS) which may mediate the initial internalization of the AI-2 to initiate the basal expression of the operon. In fact it has been shown that mutants lacking EI and EIIAglc, members of the PTS system, are unable to correctly internalize and process AI-2. This phenomenon could be explained by the involvement of the PTS system in the internalization of an initial amount of AI-2 which mediates the activation of the lsr operon. Although the whole mechanism remains unknown, the role of PTS in the AI-2 mediated QS is not surprising as it

normally mediates internalization of carbohydrates and thus reflects the metabolic status of the cell [83].

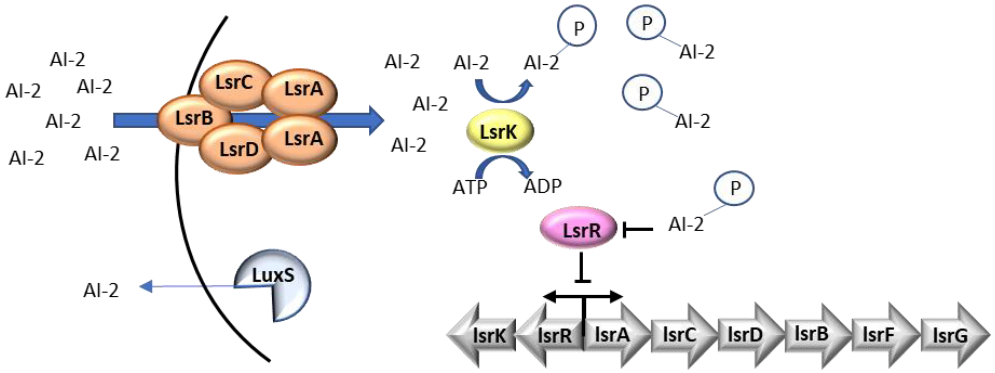


Figure 4: Schematization of AI-2 mediated QS pathway in enteric bacteria.

Additional autoinducers are 2-heptyl-4-hydroxyquinoline (HHQ) and 2-heptyl-3-hydroxy-4(1H)-quinolone (PQI), in *P. aeruginosa* [84], *cis*-11-methyl-2-dodecenoic acid (DSF), active in several strains as c-di-GMP level modulator [85], and autoinducer (AI-3) isolated from *E. coli* O157:H7. Besides promoting host colonization and damaging, AI-3 can stimulate virulence by cross-signaling with eukaryotic hormones, epinephrine and norepinephrine. QseC, a bacterial membrane bound histidine kinase sensor present in many human and plant pathogens, act as bacterial receptor by binding the hormone-like signal molecule AI-3, but, at the same time, it also acts like adrenergic receptor by interacting with epinephrine and norepinephrine [86].

QS pathways are highly interconnected. In fact, bacteria can respond to different signals and adjust their behavior in response to the combination of the autoinducers that they sense. The mechanism may have been evolved to detect not only the number of bacteria but also the composition of the population and tailor a response accordingly [87, 88]. *Vibrio* and *Pseudomonas* are perfect examples of an integrated response to several autoinducers. In fact, in *Vibrio*, the binding of CAI-2 and 3OH4CHSL to CsqS and LuxN receptors, respectively, activates the same phosphorylation cascade initiated by AI-2.

In *Pseudomonas,* 4 different mechanisms have been identified. LasIR and RhrIR recognize 3OC12HSL and C4HSL respectively. The binding of

3OC12HSL to LasR enhances AHL QS response by potentiating the expression of both receptors whereas the binding of C4HSL to RhIR only increase RhIR expression without affecting LasIR. Additionally, *Pseudomonas* also responds to PQS and IQS. Las has been proposed as one general QS regulator. It is activated by 3OC12HSL and promotes the transcription of the RhIR system among other virulence factors. At the same time C4HSL binds to RhiR promoting its own transcription. Both signals affect PQS as las promotes *pqs* operon transcription whereas RhiR inhibits it. Thus, the pathway is controlled by the ratio between the two autoinducers. However, both systems affect the PQS mediated QS. PQS promotes its own production and RhrIR activity which downregulate PQS itself. Additionally, a QscR protein which sequestrates 3OC12HSL has also been identified as AHL-mediated QS modulator [89].

Inhibiting the synthetic pathway of an autoinducer may cause QS inactivation. However, as it usually is a cytoplasmatic event, molecules able to pass the bacterial membrane are needed. Enzymatic degradation of autoinducers has been preferred as it targets their extracellular form. Lactonases and acylases catalyze AHL degradation. Lactonases break the lactone ring by targeting the ester bond [90]. Acylases act on the acyl chain by breaking the amine bond which connect them to the ring [91]. Oxidoreductases prevent the recognition of the autoinducer by its cognate receptor by altering the side chain's oxidation state [92]. All those classes of enzymes have been effective against pathogenic bacteria by restoring their susceptibility to regular antibiotics, preventing biofilm formation and spread of infections. Unfortunately, their instability limits their applications *in vivo*.

Monoclonal antibodies targeting QS have also been developed. RS2-1G9 and XYD-11G2, both inhibiting AHL activity, blocked inflammation and pyocyanin production in an *in vivo* model of infection [93, 94].

Vaccines based on an autoinducer conjugated to a vehicle have also proven to be a valid approach. In fact immunization with a viral particle based on the bacteriophage protein PP7 including a sequence from the autoinducer peptide 1 from *S. aureus* significantly reduced mortality in animal models [95]. 3-oxo-dodecanoyl homoserine lactone conjugated with BSA generated an antibody response in mice and protected them from *Pseudomonas* lung infection [96].

Natural and synthetic compounds have been proposed as candidates for the development of a class of antivirulence agents based on the inhibition of the

autoinducer/receptor interactions. 6-gingerol, isolated from *Zingiber officinale*, binds to LsrR and inhibits transcription of QS related genes, production of virulence factors and formation of biofilm, reducing severity of the infection in mice infected with *P. aeruginosa* [97]. Naringenin, a flavonoid extracted from fruits, modulates α-hemolysin mediated damages in a mouse model of MRSA infection by reducing AgrA levels, which promotes the expression of the *agr* operon involved in QS in *S. aureus* [98].

Genetic engineering may also contribute to the development of innovative quorum quenching approaches. *E. coli Nissle* has been engineered to promote its own distruction when detecting 3-oxo-C12 HSL produced by *P. aeruginosa* with release of DNAses and bacteriocins with antimicrobial activity [99]. Releasing bacteria with abnormal QS expression created by Clustered Regularly Interspaced Short Palindromic Repeats-Cas 9 interference (CRISPRi) technology into the environment to spread the construct via horizontal gene transfer and to impair the QS system in regular strains has been proposed [100]. Although this technology presents several consequences due to the release of genetically modified organisms in the environment and it is far away from being an immediate solution for the treatment of bacteria, it may represent a completely new approach were a mechanism, such as horizontal gene transfer, that bacteria use to escape antibiotics become the new tool to clear them.

2.3.3. Biofilm inhibition

Biofilm is a bacterial community surrounded by an extracellular matrix (ECM) containing exopolysaccharides (EPS), proteins and nucleic acids. Physical forces and adhesion proteins mediate bacterial attachment to a surface and initiate the organization of aggregates which will build the first layer of biofilm [101, 102]. The adhesion is followed by a maturation phase with the production of the ECM components which supports the formation of a complex 3D structures. Ultimately, the biofilm will be disrupted causing the spreading of bacteria and propagation of the infection.

As biofilm forming community, bacteria obtain several advantages. Among those, resistance to antibiotics is particularly significant as it makes biofilm hard to eradicate and thus a serious complication for the complete clearance of infection. Resistance is a multifactorial phenomenon partially due to the different metabolic activity of biofilm embedded cells compared to the

planktonic status [103]. Within biofilm, growth is not a priority for bacteria which significantly suppress their metabolic activity. As antibiotics target vital processes in bacteria linked to their duplication, dormant cells become immune to the treatment and may eventually reactivate in absence of the antibiotic, becoming pathogenic [104, 105, 106, 107]. ECM is also essential for resistance as it actively hinders antibiotics penetration and support horizontal gene transfer with consequent spreading of resistance [108].

The role of biofilm in infection has been underestimated for many years. Persistent cells cause chronicity of infections which can be fatal, especially for immunocompromised patients [109]. The advancement in implant design and application also increased the spread of biofilm-related infections as it provides an additional surface suitable for contamination [110]. Thus, preventing biofilm formation or promoting its dispersal should become an integral part of the infection treatment.

Albeit QS has been recognized as one of the main biofilm regulators and its inhibition may also be associated, among the other effects, to biofilm reduction, specific biofilm regulators have also been discovered and targeted.

C-di-GMP and pppGDPpp are GTP derivatives which contribute to biofilm formation in several bacteria [111]. pppGDPpp acts as transcriptional factor for genes involved in biofilm formation and development of persistent cells [112]. C-di-GMP controls biofilm initiation by downregulating mobility and upregulating mechanism of adhesion and production of ECM [113]. Hampering these two regulators could strongly affect biofilm and consequently restore bacterial susceptibility to regular antibiotics and host immune system. C-di-GMP is synthetized by diguanylate cyclase and degraded by phospohodiesterases[114]. Interfering with the c-di-GMP synthesis by inhibition of diguanylate cyclase has been shown to be active against *in vitro* biofilm formed by *P. aeruginosa* and *Acinetobacter* [115]. Promoting c-di-GMP degradation by overexpression of phosphodiesterases in *P. aeruginosa* reduced biofilm formation in a mouse model of implant related infection [116].

Dispersal of preformed biofilm will expose bacterial cells to the action of antibiotics. Enzymes for the degradation of matrix components cause biofilm disruption in several models of biofilm and are particularly efficient when used in combination with antibiotics [117, 118, 119]. Antibiofilm molecules are also available in nature as organisms, including bacteria, have evolved

self-defense mechanisms to contrast biofilm formation by invasive species. Biofilm inhibitors have been identified in *Vibrio* and *Pseudomonas* [120, 121]. EPS273, isolated from *P. stutzeri* 273, prevents the formation of stable biofilm in *Pseudomonas* by altering the pyocyanin pathway [122]. Nitric oxide has been found active against biofilm in several strains [123]. Although the numerous side effects restrict its application in clinic, the administration of picomolar doses of nitric oxide have been proven to be safe and still efficient in biofilm eradication. Biofilm of *P. aeruginosa* has been cleared by a chimeric drug containing cephalosporine linked to a NO donor, released after cleavage of the β-lactam ring by β-lactamases, *in vitro* model [124]. The number of bacterial colonies in a chronic model of biofilm-related infection have been considerably reduced by effect of gallium. In fact, this metal is accumulated by bacteria due to its similarity to iron but, as it cannot replace iron functionality, leads to cell death [125].

Antibiotics traditionally used for the treatment of slow-growing species find their application in the treatment of biofilm related infection as they may target persisters. In fact antibiofilm agents have the potential to destabilize biofilm and thus restore susceptibility to antimicrobial compounds. A screening campaign aiming to evaluate the effect of small molecules, already approved as drugs, in combination with colistin for the treatment of *P. aeruginosa* led to the identification of two compounds, auranofin and clomiphene citrate, which together with colistin were active against the planktonic form and mature biofilm formed by laboratory and clinical strain [126]. Co-administration of a new class of D-enantiomeric antimicrobial peptide (AMP), acting as (p)ppGpp repressors, with ciprofloxacin, imipenem or trombomycin has improved the effectiveness of the antibiotic alone. Additionally, it contributed to successful eradication of mature biofilm formed by important pathogens such as *K. pneumoniae*, *A. baumanii* and *S. enterica* [127].

2.4. High throughput screening

High throughput screening (HTS) is a standardized procedure which allows rapid assessment of thousands of compounds for the identification of few candidates which are suitable for further development. A general workflow of a screening campaign includes the primary screening where compounds are typically screened in singlet or duplicate at one concentration followed

by a second round of screening (secondary screening) where the positive hits are confirmed and tested for dose response. Follow-up studies are then performed to further characterize the hits and confirm their activity to provide most potential compounds for further development [128].

A screening campaign starts with the assay development which consists of designing and validating a sensitive and simple assay suitable for automation. An ideal assay should only include addition and incubation steps before the read-out as those are easier to automate.

The nature of the assays is strictly related to the biological phenomenon of interest. However, as general rule assays are divided into biochemical or cell-based assays. Biochemical assays are target-based assays, which monitor the interaction between a test compound and a target which is known to be relevant in a disease. Those assays have high throughput and are less time consuming. However, they are not always physiological and thus the hits may not be active when tested in cellular environment.

Cell-based assays measure the effect of test compounds typically on an entire pathway in physiological conditions. The target is very often initially not known, and several studies are required to identify it and determine the mode of action. These kinds of assays are thus very suitable for the identification of new targets to be further investigated or new classes of drugs. However, the throughput is usually lower and they are more complex and time consuming than target-based assays [129].

Additionally, virtual screening is also becoming more popular due to the advancements in technology. Indeed, it allows fast screening of large compound databases providing restricted numbers of hits to be tested *in vitro*. This approach includes target-based and structure-based virtual screening. The first option requires the available structure of the target which is used to screen virtual libraries; the structure-based procedure instead is based on the knowledge of bioactive compound(s) which are used as templates to identify analogues which share the same active motif [130].

Since the aim of a screening campaign is to select few active molecules out of large number of inactive compounds, quality control is critical. Assays for HTS must be carefully optimized and validated to ensure the robustness and reproducibility. Assay quality is typically assessed by so-called assay quality parameters calculated from controls. For example, signal-to-background (S/B) ratio is normally used to determine the separation between the

maximum and minimum controls. Z' factor is a more useful parameter as it considers not only the mean of the controls but also the SD as indicator of the variability. The value is comprised between 0 and 1 with values ≤0.5 indicating a poor-quality assay and values ≥ 0.5 indicating good quality [131].

Various detection technologies are used in HTS assays to quantify the effect of a compound on the target or pathway being studied. The selected technology may influence significantly the sensitivity of the assay. Fluorescence is based on an ability to emit the absorbed electromagnetic radiation. It is widely used in HTS although it is quite susceptible to interference e.g. from auto fluorescent compounds [132]. To reduce the disturbance, different approaches have been developed, for example, by aiming to monitor the proximity of two fluorescent probes rather than directly detecting fluorescence [133]. The fluorescence resonance energy transfer (FRET) is based on the interaction of an acceptor and donor molecule which are close in space. The donor emits at the wavelength which is absorbed by the acceptor. Thus, by exciting at the donor wavelength and reading at the acceptor emission wavelength, it is possible to determine the proximity of the two probes [134]. As evolution of FRET, time-resolved fluorescence (TRF) uses the same principle but with a donor which will have a longer fluorescence decay time than the interfering compounds [135].

The term bioluminescence indicates the production of light as product of a chemical reaction catalyzed by enzymes named luciferases. Bacterial luciferase has two domains encoded respectively by *luxA* and *luxB* which are organized into the luxCDABEG operon. *LuxC*, *LuxD* and *LuxE* encode for an enzymatic complex responsible for the synthesis of an aliphatic aldehyde whereas *luxG* encodes for a flavin reductase. The long fatty acid aldehyde is oxidized by a reduced flavin mononucleotide with consequent production of light [136]. The eukaryotic reaction involves different substrates. The most common eukaryotic luciferase isolated from firefly catalyzes the conversion of luciferin into oxyluciferin in an ATP-dependent manner with production of light as a side product [137]. As it is an ATP dependent reaction, this method is particularly suitable for the study of kinases or cellular viability. The luciferase gene can be inserted into a plasmid under control of a promoter of interest and cloned into a host strain to generate a bioreporter strain. The emission of light will be proportional to the level of activation of the promoter after the treatment with a compound of interest [138].

Additionally, platforms like FRET have been developed also for luminescence. Alphascreen technology involves a donor or an acceptor molecule linked to beads. When the donor is excited at 680 nm it promotes the conversion of oxygen into singlet oxygen which can travel for approximately 200 nm before decaying. If the acceptor is within that distance the singlet oxygen initiates a chemiluminescent reaction on the acceptor which emits between 520 and 620 nm. In this case the interference by other compounds is significantly reduced thanks to the range of selected wavelengths [139].

3. Aims of the study

Since QS has been highlighted as one of the main regulators of bacterial virulence, the inhibition of enzymes involved in QS establishment has become one of the most promising strategies to reduce bacterial pathogenicity. The aim of this thesis was the development of a screening approach for the discovery of LsrK inhibitors. LsrK has been described as one of the key enzymes in the AI-2-mediated QS in gut enteric bacteria. In fact, once DPD, the precursor of AI-2, is internalized by the Lsr transporter, it is phosphorylated by LsrK and only the phosphorylated form can bind the repressor LsrR, enhancing the response to QS signals. Additionally, it has been demonstrated that mutant bacteria which do not express LsrK, are unable to establish AI-2-mediated QS [140], qualifying the enzyme as potential target for the development of a new class of antivirulence agents.

Specifically, the aims were:

- design, evaluation, optimization and validation of HTS-compatible assay for screening chemical libraries to identify LsrK inhibitors (I)
- screening of chemical libraries by the LsrK inhibition assay. Three chemical libraries were selected: a commercially available library, a small set of commercially available compounds selected by virtual screening and a small set of DPD analogues. (I, II, III)
- follow-up studies of the hits selected by primary screening including IC_{50} estimation, binding assays, and cell-based assays (I, II, III)
- development of a luminescence-based bioreporter strain to confirm the activity of positive hits selected by target-based assay as well as to allow fast and simple cell-based screening of chemical libraries to identify AI-2-mediated QS inhibitors (IV).

4. Materials and methods

This section includes a summary of the materials and methods used in the studies. The detailed procedures can be found in the original publications (I-IV).

4.1. Bacterial strains and growth conditions

For the over-expression of LsrK, *E. coli* strain MET1158 [*E. coli*, amp resistance, BL21 (DE3) luxS-, with pMET1144 (lsrK-His in pET21b)] was kindly donated by Prof. Karina Xavier (Instituto Gulbenkian de Ciencia, Portugal) [141]. The strain was grown in YTPG (yeast, tryptone, phosphate buffer and glucose) medium supplemented with 100 μg/ml ampicillin at 18°C (250 rpm) until exponential phase and protein expression was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 9 h at 22°C (250 rpm). LsrK is purified by Ni-nitriloacetic acid chromatography.

E. coli LW7 pLW11 and *E. coli* pBAC-LacZ, used in the AI-2 mediated QS interference assay, were kindly provided by Prof. William Bentley (University of Maryland, USA) and Keith Joung (Addgene plasmid # 13422), respectively. Both strains were grown at 37 °C in lysogenic broth (LB) supplemented with 100 μg/ml ampicillin and 50 μg/ml kanamycin for *E. coli* LW7 pLW11, or with 12.5 μg/ml chloramphenicol for *E. coli* pBAC-LacZ.

For study IV, *E. coli* JW5503 and *E. coli* ATCC 25922 were obtained from the NBRP-*E. coli* collection at the National Institute of Genetics (NIG, Japan) and the American Type Culture Collection (ATCC) via Microbiologics Inc. (St. Cloud, MN), respectively. *E. coli* JW5503 and *E. coli* ATCC 25922 were transformed with pET-Plsrlux and grown at 37 °C in LB supplemented with 100 μg/ml kanamycin and 100 μg/ml ampicillin or with 100 μg/ml ampicillin respectively.

4.2. Compound libraries and their screening for LsrK inhibition

For study I, the MicroSource Spectrum library containing known drugs (50%), natural products (30%) and other bioactive compounds (20%) for a

total of 2000 compounds, was purchased from FIMM. The library was used for the assay validation. Compounds were pre-plated by Echo acoustic dispenser (Echo, Labcyte Inc., CA, USA) into 384-well plates at 50 μM concentration in singles. The set of 107 positive hits selected for secondary screening were obtained from FIMM and pre-plated at 10 μM in singles to be re-tested against LsrK and at 50 μM in singles to be tested against glycerol kinase. The 22 compounds which were selected by secondary screening for dose-response assay were pre-plated at FIMM at 6 concentrations (25-0.39 μM). Finally, harpagoside, rosolic acid, agaric acid and aurin tricarboxylic acid were purchased from Sigma (USA) for further studies.

For study II, a set of 104 compounds pre-selected by virtual screening was acquired from FIMM and pre-plated by an Echo acoustic dispenser in 384-well plates in triplicate at 2 concentrations (20 and 200 μM) for the LsrK inhibition assay. The positive hits were used as template for the selection of analogues in the MolPort database by catalogue-search approach. The prioritized analogues were purchased from different vendors and tested at 200 μM in the LsrK inhibition assay and the positive hits were selected for dose-response.

In study III, two sets of DPD-inspired compounds including respectively 19 and 29 compounds were synthetized by the Medicinal Chemistry department of Taros Chemicals GmbH&Co (Dortmund, Germany). Compounds were dissolved in DMSO and plated in triplicate at 200 μM in 384-well plate.

In study IV, a library of 91 compounds designed to target the ATP binding pocket of DNA-gyrase was synthetized and provided by the Medicinal Chemistry Department of the Faculty of Pharmacy at the University of Ljubljana (Ljubljana, Slovenia). Compounds were dissolved in DMSO and plated in 384-well plate in triplicate at a final concentration of 100 μM for the primary screening against LsrK. The same set was plated in triplicate in 96-well plate at 100 μM for the bioreporter assay

4.3. LsrK assay selection

To develop a LsrK inhibition assay, two commercially available kit based on ATP depletion and ADP formation, respectively, were tested.

4.3.1. Kinase-Glo Max Kinase Luminescence assay

300 nM LsrK and 300 μM DPD (Carbosynth, UK) in assay buffer [25 mM triethanolamine (TEA), 200 μM MgCl2, 0.1 mg/ml bovine serum albumin (BSA), 0.01 % Triton X-100] were added to a 384-well plate (Greiner). 100 μM ATP were added as last component to start the reaction. The total assay volume was 20 μl. The reaction was kept at RT for 15 minutes. According to manufacturer's instruction, 20 μl of kit's components were added to the plate. After 15 min, the luminescence was recorder at Varioskan LUX plate reader (Thermo Fisher Scientific, Finland)

4.3.2. ADP-Quest

300 nM LsrK, 300 μM DPD and 100 μM ATP in assay buffer [25 mM triethanolamine (TEA), 200 μM MgCl2, 0.1 mg/ml bovine serum albumin (BSA), 0.01 % Triton X-100] were added to a 384-well plate. After 15 min incubation at RT, 10 μl of Reagent A and 20 μl of Reagent B were added to the plate and the mixture was incubated for 30 min. Then fluorescence was measured at Varioskan LUX plate reader.

4.4. LsrK inhibition assay

The selected LsrK inhibition assay was used in study I, II, III and IV to identify compounds with inhibitory activity on LsrK. Compounds were diluted in assay buffer [25 mM TEA, 200 μM $MgCl_2$, 0.1 mg/ml BSA, 0.01 % Triton X-100] and plated into a 384-well plate. More details about concentrations and replicates are presented in paragraph 4.2. Then, 300 nM of purified protein and 300 μM DPD diluted in assay buffer were added and the assay was carried out as described in paragraph 4.3.1.

4.5. Glycerol kinase inhibition assay

Glycerol kinase inhibition assay was used in study I, II and III to preliminarily evaluate the selectivity of the positive hits identified by LsrK

inhibition assay. The set-up described in the paragraph 4.3. was used with 300 nM glycerol kinase and 300 μM glycerol.

4.6. Thermal shift assay

In study II thermal shift assay was used to confirm the target engagement of the positive hits. 25 μl of a reaction mixture containing 2.5 μl of 1.73 μM LsrK, 2.5 μl of SYPRO orange dye and 10 μl of compounds at 5 concentrations ranging from 0 to 300 μM in assay buffer was plated on a 96-well plate. A sample containing LsrK and 1μM ATP was used as positive control. The assay was performed on a real-time polymerase chain reaction (PCR) thermocycler (Stratagene MX3005P QPCR System, Agilent Technologies, CA, USA) applying a gradient heating of 0.066 $°Cs^{-1}$.

4.7. AI-2 mediated QS interference assay based on β-galactosidase activity

In study I, a cell-based AI-2 mediated QS interference assay based on *E. coli* LW7 pLW11 was performed to evaluate QS inhibition activity of rosolic acid, harpagoside and aurin tricarboxylic acid. The strain contains the pLW11 plasmid where the β-galactosidase gene is under control of *lsrACDBFG*, a QS responsive promoter. An overnight culture was diluted in fresh lysogenic broth (LB) and grown at 30°C till exponential phase. For the assay, a bacterial suspension containing 10^9 CFU/ml bacteria (2×final concentration) in PBS with or without 100 μg/ml phe-arg β-naphtylamide dihydrochloride (PAβN) was prepared (DPD-). Half of the suspension was supplemented with 40 μM DPD (2×final concentration) (DPD+). The compounds were diluted in PBS with and without 100 μg/ml PAβN to a final concentration of 100 and 20 μM (2×final concentration) and 50 μl for each sample were plated in triplicate in 96-well plate. DPD- suspension was added to half of the plate while the other half was filled up with DPD+ suspension. After 2 hours incubation at 37 °C under shaking, abs at 600 nm was measured with the Multiskan GO plate reader (Thermo Fisher Scientific, Finland). The plate was frozen overnight. The following day, detection mix including [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 36 mM β-mercaptoethanol, 6.7% PopCultureTM reagent, 1.1 mg/ml 2-

nitrophenyl β-D-galactopyranoside (ONPG)] was added to the plate and the plate was incubated for 2 hours before reading the absorbance at 420 and 550 nm.

4.8. Luminescence-based AI-2 mediated QS interference assay

4.8.1. Plasmid construction

A luminescence–based bioreporter strain for the identification of AI-2 mediated QS inhibitors was developed in study IV. The plasmid pLW11, containing β-galactosidase gene, under control of *lsr* promoter, was extracted from *E. coli* LW7 pLW11 and used as template for the amplification of the lsr promoter. The PCR product was cloned into pET-PesaRlux[142], previously digested the *esaR* promoter. The pET-Plsrlux construct was transformed into Subcloning efficiency DH5*α* strain and then transferred into *E. coli* JW5503 or *E. coli* ATCC25922.

4.8.2. Assay development and screening campaign

E. coli JW5503 pET-Plsrlux was grown overnight and diluted 1:50 in fresh LB supplemented with the appropriate antibiotics. Bacteria were grown at 30 °C under shaking till exponential phase. Compounds were diluted at 200 μM (2×final concentration) in LB and 50 μl were plated in triplicate into 96 well-plate (View Plate, PerkinElmer Winter St., Waltham, Massachusetts, USA). For negative and positive control wells, 50 μl of LB and 50 μl of LB supplemented with 4% glucose (2×final concentration), respectively, were plated in triplicate. 50 μl of a bacterial suspension containing 1×10^8 CFU/ml (2×final concentration) were added to the plate. The plate was incubated at 37 °C for 3 hours under shaking and then Abs600 and luminescence were measured with Varioskan LUX plate reader.

Compounds with QS inhibition ≥ 80 % and growth inhibition ≤ 40 % were selected for dose-response experiments and tested at 7 concentrations according to the same protocol.

4.9. Data analysis

S/B, SW and Z′ factor, quality parameters typically used for assay development and HTS, were calculated according to the following equations to evaluate assay performance [143,144]:

$$S/B : \mu M/\mu m \quad (1)$$

$$SW : (\mu M-\mu M)/SQRT[(\sigma M^2)+(\sigma m^2)] \quad (2)$$

$$Z' : 1 - [(3*\sigma M+3*\sigma m)/(|\mu M|-|\mu m|)] \quad (3)$$

In the equations μM and μm represent the average for the maximum and minimum signals, respectively, whereas σM and σm represent the standard deviation. Z′ factor ≥ 0.5 indicates a good quality assay.

The inhibition % was calculated according to equation 4 where X is the mean of the detected luminescence for the sample and XM and Xm represent respectively mean of the detected luminescence for the maximum and minimum control:

$$\text{Inhibition \%} : 100 \times [(X-Xm)/(XM-Xm)] \quad (4)$$

In study III the promiscuity index, PCIdx, based on PubChem activity profile, was calculated according to the following equation [145]:

$$PCIdx = N(active)/N(tested) \quad (5)$$

N(active) is the number of assays reported in PubChem where the analyzed compound has been classified as active whereas N(tested) is the total number of assays reported.

The inhibition % for AI-2 mediated QS interference was calculated as follows

$$\text{Inhibition\%} = 100 - \text{remaining activity \%} \quad (6)$$

Wherein remaining activity % for the β-galactosidase based assay corresponds to [146]:

$$\text{Remaining activity \%} = 100 \times [MUs(DPD+) - MUc(DPD-)]/[MUc(DPD+) - MUc(DPD-)] \quad (7)$$

And for the luminescence-based assay corresponds to:

$$\text{Remaining activity \%} = 100 \times [(X-Xm)/(XM-Xm)] \quad (8)$$

In the equation 7, MUs and MUc respectively correspond to the _ galactosidase activity expressed in Miller Unit (MU) calculated for the sample and the DMSO control, with (DPD+) and without DPD (DPD-).

In the equation 8, X is the luminescence detected for the sample and XM and Xm are respectively the mean of the detected luminescence for the minimum and maximum controls.

5. Results and discussion

This chapter summarizes the main results of the studies which are presented in more detail in the attached publications (I-IV).

Study I, II and III focused on the development of an enzyme-based assay and its application during screening campaigns to discover LsrK inhibitors. Overall, the work significantly improved the knowledge on LsrK and provided interesting compounds which can be used as starting points for the development of a new class of antivirulence agents based on LsrK inhibition. Article IV presents the development of a new bio-reporter strain for the identification of AI-2 mediated QS inhibitors which can contribute to the discovery of new classes of antivirulence agents.

5.1. Target-based screening

5.1.1. LsrK assay development

LsrK is a bacterial kinase belonging to FGGY sugar kinase family which mediates an essential step in the AI-2 mediated QS in gut enteric bacteria [147]. In fact, the AI-2 phosphorylation by LsrK is essential for de-repressing the *lsr* promoter and beginning the QS cascade [140]. The LsrK crystal structure showed that the enzyme has two domains [148]. The domain II binds to ATP whereas the domain I binds to HPr, a phosphate carrier which contributes to phosphorylation of sugars internalized by PTS system. This further confirmed the essential role of LsrK in QS as it is subjected to a fine regulation. In fact, QS establishment depends on bacterial cellular density and the complex HPr/LsrK acts as a sensor. During the exponential phase, the sugar import is particularly intense so that HPr will be mainly present in the unphosphorylated form with an inhibitory effect on LsrK and consequently, on QS. The phosphorylated form of HPr is dominant in the stationary phase, when the population density is high and thus import of sugar is reduced. P-HPr correlates to an active form of LsrK, thus response to QS will be enhanced in stationary phase [149]. Protein-protein interaction appears thus to be a natural mechanism to inhibit LsrK and silence QS. Therefore it is legit to think that LsrK inhibition may lead to QS inactivation and consequent loss of virulence.

However, no systematic screening approach has previously been carried out against LsrK. Earlier reported assays to identify LsrK inhibitors include thin layer chromatography (TLC) with [γ-^{32}P] or the detection of ATP consumption [150, 151]. However, radioactivity-based assays find limited applications in large screening campaigns due to the use of radiolabeled materials and the reported assays based on ATP consumption were not aiming to screen chemical libraries for LsrK inhibitors but rather to evaluate the effect of DPD modifications on its processing [164].

To identify LsrK inhibitors by HTS, we designed a luminescence-based LsrK inhibition assay in 384-well plate format. As ATP-based methodologies are widely used to study kinases, two commercially available kits were compared: Kinase-Glo Max Luminescent kinase assay (Promega), based on the conversion of the remaining ATP in the reaction mixture into light, and ADP-Quest (DiscoverX), based on the conversion of ADP, formed during the reaction, into a fluorescent probe (Table 4). Read-out stability and DMSO tolerance were determined and compared for both kits. Sensitivity and automation-compatibility were also taken into account.

Table 4: Comparison of Kinase-Glo Max Luminescence kinase assay and ADP-Quest kit

Kinase-Glo Max Luminescence kinase assay	ADP-Quest
• ATP depletion method • Sensitive (S/B ≥60) • ATP up to 500 µM • Signal stable up to 5 hours • DMSO tolerance up to 5% • easily automatable	• ADP formation • Low sensitivity (S/B = 4) • ATP up to 200 µM • Signal stable up to 1 hour • DMSO tolerance up to 5%

Due to the high sensitivity and stability, the final assay was based on Kinase-Glo Max Luminescence kinase assay, according to the following scheme (Figure 5):

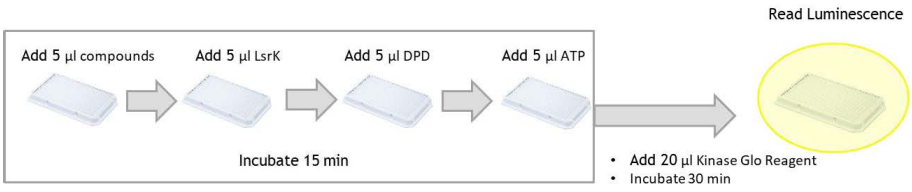


Figure 5: Schematization of the LsrK inhibition assay protocol.

5.1.2. Screening campaigns

The described LsrK inhibition assay was used to perform screening of 3 chemical libraries

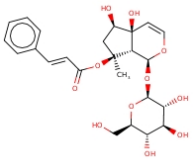
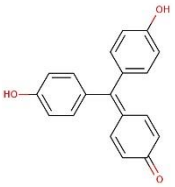
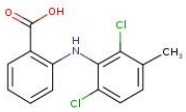
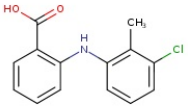
In study I, results from the screening campaign on the MicroSource Spectrum library are reported (Table 5). The library contains 2000 molecules including synthetic compounds, which have reached clinical trials phase or are approved drugs, and a variety of natural products ensuring high structural and activity diversity. The primary screening yielded 107 compounds which were tested in a glycerol kinase inhibition assay for preliminary assessment of selectivity. The same set was also re-tested in the LsrK inhibition assay at a lower concentration to estimate potency. The results of the two tests were compared and 47 compounds were selected for follow-up studies. Among those, 25 were excluded as they were reported in literature as unspecific inhibitors or antibacterials. A final set of 22 compounds was tested for dose-response (I, Fig 3). Among those, rosolic acid and harpagoside confirmed their activity as QS inhibitors in a cell-based AI-2 mediated QS interference assay in combination with efflux blocking PAβN [152].

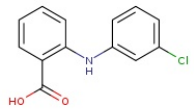
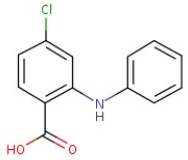
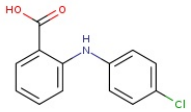
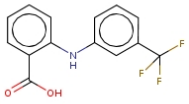
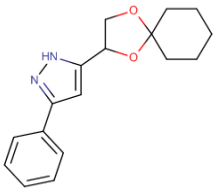
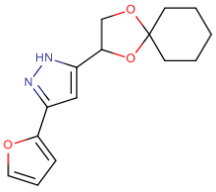
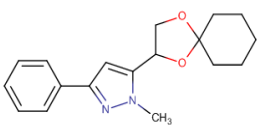
In study II, the design of a 3D LsrK homology model and its application for the virtual screening of a commercially available database is described. The model was built using as a template the crystal structure of xylulokinase and glycerol kinase as they were found to be LsrK homologs by PSI-BLAST sequence analysis [153]. The model was validated by Ramachandran plot, ModVal, ERRAT and the RMSD (root mean square deviation) with templates and used for the virtual screening of the FIMM database including 132 566 compounds. Compared to *in vitro* screening, virtual screening offers the chance to rapidly explore chemically diverse databases and select compounds with high probability to bind the target protein. 104 compounds were selected and tested against LsrK in an inhibition assay resulting in 2 active compounds, meclofenamic acid and IOX1, which were used as templates for the selection of analogues. The second round of screening resulted in a total of 6 hits which belong to the meclofenamic acid series. 5 among those were confirmed to bind to LsrK by thermal shift assay (Table 5).

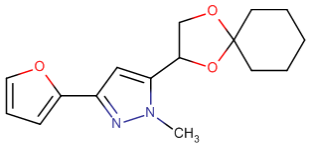
In study III, the synthesis and biological evaluation of DPD analogues is presented. The advantage of the ligand-based approach is the selectivity

ensured by the targeting of the unique enzymatic catalytic site. However, since DPD is an unstable molecule which exists as several structures due to cyclization [154], the design and synthesis of analogues is complex. Differently than previously exploited analogues [155, 156, 157, 158] the synthetized analogues support modification on the diketo-moiety which is incorporated into the heteroaromatic ring to increase stability of the molecule. 4 compounds, belonging to the pyrazole-containing series were active against LsrK with an IC_{50} ranging between 119 and 475 μM (Table 5).

Table 5: Active compounds and their IC_{50} identified by LsrK inhibition assay in studies I, II and III. Data are presented as average ± SD from two independent experiments (n=4)

Study	Compound ID	Structure	IC_{50} (μM)
I	Harpagoside		10 ± 0.1
	Rosolic acid		1±0.2
II	Meclofenamic acid		178±11
	3		70 ±2

	4		119±15
	5		30±8
	6		141±8
	7		67±3
III	12a		475 ± 11
	12b		384 ± 15
	13a		119 ± 3

	13b		284 ± 19

The random screening provided the best hits which also showed activity as QS inhibitors in the cell-based assay. To our knowledge, rosolic acid and harpagoside are currently the most promising scaffolds for the development of a new class of LsrK inhibitors to be used as antivirulence agents. But, the high IC_{50} values and the need of efflux pump blocking PAβN hamper the interest of these compounds for *in vivo* studies. However, additional studies may provide a better understanding of the molecular interaction between these compounds and LsrK, and lead to the identification of a functional structural motif. Chemical support will also be needed to improve potency and to enhance cellular accumulation.

Virtual screening approach provided 6 active compounds which all belong to the meclofenamic acid series highlighting the presence of a significant structural motif. The compounds may act as ATP competitors as their potency increases at lower ATP concentrations. The ligand-based approach resulted in 4 positive hits from the pyrazole containing analogues with considerably low potency. Although those compounds are too weak to be considered as candidates for the development of antivirulence agents, they offered an important hint to better understand the interaction between LsrK and DPD and the contributions of different substituents to the binding. SAR studies performed on the positive hits from virtual screening and ligand-based approach highlighted the importance of Thr275 which is located near the LsrK binding site. In fact, the activity of the most effective compound from study II was explained by the formation of H bond with Thr275 and surrounding amino acid near the binding site (II, Fig S15). The same interaction was observed for compound 13a (III) which is the most active compounds among the DPD analogues described in study III (III, Fig 5). Active compounds from both studies also showed interaction with Phe276 and Gln278 although their potency was inferior to compounds interacting with Thr275. Thr275 may thus have an essential role for the catalytic activity of LsrK. Study III also highlighted the stabilizing role of acetals which form electrostatic interactions with LsrK binding site whereas diols form repulsive interactions.

Overall these considerations broaden our knowledge on LsrK and may support the design of new compounds with improved activity and better pharmacokinetic properties for *in vivo* studies and clinical applications.

5.2. Cell-based assay development and screening

5.2.1. Development of a bioreporter strain for QS inhibition studies

Cell-based assays are essential screening tools to confirm the activity of hits selected by target-based assays in more physiological conditions. They can also be used as direct screening platforms for the identification of compounds which are active on the pathway of interest even if targeting an unknown component.

Previously reported cell-based assays for the identification of AI-2 mediated QS inhibitors include *E. coli* LW7 pLW11, a bioreporter strain based on β-galactosidase expression in response to QS activation, and AI2-QQ.1, a bioreporter strain based on the toxin/antitoxin system. In the first example, β-galactosidase is under control of the *lsr* operon and will be expressed when the QS system is active. The enzyme can be quantified by following the formation of a colored product, derived from the cleavage of ONPG. A QS inhibitor will prevent β-galactosidase expression and consequently no colored product will be detected [146]. The length and complexity of the protocol, together with the need of a secondary control strain to verify the interference of the tested compounds with β-galactosidase activity, are significant limitations for the use of this set-up for screening. The AI2-QQ.1 strain expresses the lethal gene *ccdb* from *E. coli* in response to the activation of a QS responsive promoter. Thus, when QS is active, the protein is expressed, and the cell dies whereas, the addition of QS inhibitors, will result in a viable cell [159]. It also requires a control strain to assess compound toxicity. Additionally, both set-ups need the addition of exogenous DPD with an increase in the cost and artificiality of the system.

In study IV, a new cell-based assay for the identification of AI-mediated QS inhibitors is presented. The concept is based on the expression of luciferase under control of *lsr* promoter. The construct pET-Lsrlux was generated starting from the commercially available plasmid pET-pEsaRlux by replacing the EsaR promoter with the *lsr* promoter sequence. The new

plasmid was cloned into *E. coli* JW5503, an efflux pump defective strain. In fact, as one of the main limitations in the identification of new antibiotics is the low intracellular concentration due to the expulsion by the efflux pump system, this strain will allow the intracellular accumulation of test compounds and thus facilitate the identification of new hits [160]. Compared to previously reported assays for the identification of AI-2 mediated QS inhibitors, the new bioreporter strain produces endogenous DPD so that no external addition is needed reproducing more physiological conditions and reducing the costs. It also allows the simultaneous identification of toxic compounds as detection of luminescence can be coupled with detection of absorbance to determine viability so that no additional tests are needed for this purpose.

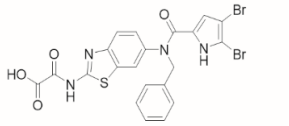
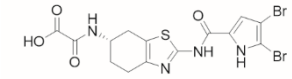
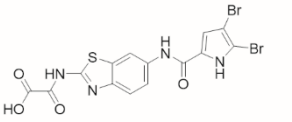
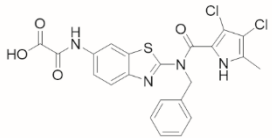
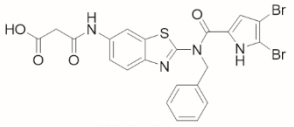
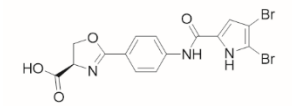
In the assay development phase, optimal bacterial concentration, effect of DMSO and read-out stability were evaluated. The finalized protocol included a starting bacterial concentration of $5^{\wedge}10^{7}$ CFU/mL and an incubation time of 3 hours. In fact, the Z' factor reached a value of 0.92 after 3 hours and remained stable (≥0.84) up to 7 hours ensuring an acceptable time window to detect the signal. The DMSO concentration must be kept ≤2% as higher concentrations affect the luminescence.

5.2.2. Screening campaign

To validate the assay, we tested 91 compounds designed to target the ATP binding site of DNA gyrase [161]. Our hypothesis was that these compounds would thus be capable to bind also to the LsrK ATP binding site. The entire set was tested in the LsrK inhibition assay and on the new bioreporter assay in parallel and the results were compared.

IC_{50} for LsrK was determined for 29 compounds, ranging from 8 to 147 μM. Among those, 6 hits were confirmed to be active as inhibitors in the cell-based AI-2 mediated QS interference assay (Table 6). UL-03 and UL-21 were the best compounds with an IC_{50} in the submicromolar range in the QS inhibition assay (IV, Fig S4). However, all the compounds shower higher potency in the cell-based assay. This might be due to structural differences observed in the catalytic domains of LsrK from *S. thyphimurium*, used in the target-based assay, and LsrK from *E. coli* used in the AI-2 interference assay, which affects the interaction with small molecules [162].

Table 6: Hits and their IC_{50} determined by LsrK inhibition assay and AI-2 mediated QS interference assay. Data are presented as average ± SD from two independent experiments (n=3)

Compounds	Structure	IC_{50} LsrK (μM)	IC_{50} QS (μM)
UL-05		18± 2	10± 0.4
UL-06		147± 12	2 ±0.2
UL-03		18 ±2	0.6± 0.1
UL-19		29±2	1.0± 0.1
UL-21		15± 1	0.4 ±0.2
UL-09		78± 1	17± 2

The cell-based AI-2 mediated QS interference assay also identified 18 hits which were not active on LsrK. Those compounds may target other components involved in the pathway and thus be a potential starting point for the development of a new class of QS inhibitors. However, additional investigations are needed to identify the target and clarify the mode of action as well as to improve their cell accumulation. In fact, none of the

compounds showed activity when tested on *E. coli* ATCC 255922 with regular efflux pump activity.

Although the study did not provide any suitable candidate for future clinical applications, it described the design and validation of a new effective tool for QS inhibitors discovery as the assay can be easily scaled-up and automated to support larger screening campaigns. Furthermore, it proved the efficacy of the new format to support the confirmation of hits selected by target-based assay as well as the identification of new molecules active on the pathway of interest.

6. Conclusions

Antivirulence agents have the potential to revolutionize the treatment of bacterial infections by targeting virulence factors rather than viability and thus reducing selective pressure for resistant mutants.

The aim of this thesis was to support the discovery of new QS inhibitors by targeting LsrK, a key enzyme for the processing of autoinducer-2 in enteric bacteria. Study I, II and III report data related to three screening campaigns performed for the identification of LsrK inhibitors. The major difference among the three studies is the nature of the screened libraries. In fact, for study I, the chosen library was unrelated to LsrK and was selected to investigate structurally diverse compounds aiming to isolate meaningful structural motifs for LsrK inhibition. In study II, the set of compounds for the screening was selected by virtual screening using a 3D LsrK model to maximize the chance of interaction with the target enzyme by using a target-based approach. The compounds screened in study III were synthetic DPD analogues which were designed to target the LsrK substrate binding pocket. Although none of the studies provided significant hits for future follow-up studies and drug design, they broaden our knowledge on LsrK and its mode of action by highlighting the significance of Thr275 for the enzymatic activity of LsrK and thus providing interesting hints for future screening campaigns and drug design. Additionally, rosolic acid and harpagoside identified as LsrK inhibitors in study I, were also effective on the AI-2 mediated QS interference assay proving that LsrK inhibition may indeed lead to the inactivation of the pathway.

Aiming to support the discovery of AI-2 mediated QS inhibitors, study IV described the assay development and validation of a new AI-2 mediated QS interference assay based on the emission of luminescence in response to QS activation by a genetically modified *E. coli* JW5503. The assay has been proven to be useful as complementary assay to confirm the activity of compounds selected by target-based assay but also as independent screening tool for a fast and easy identification of QS inhibitors. In fact the same set of 91 compounds, chosen for their potential to target the ATP binding pocket of LsrK, were tested in both the target-based and cell-based assay providing two sets of active hits. The first set includes 6 compounds which were also active against LsrK and thus were confirmed to be QS inhibitors by interfering with the kinase activity. The second set includes 18 compounds which were exclusively active in the cell-based assay and may thus target other elements in the pathway which play an essential role and deserve to be further investigated.

Based on the overall results of these studies, future research on LsrK inhibitors should be addressed to better understand the mode of action by using the found inhibitors as starting points. A synergistic effort among bioinformatic, chemistry and biology will be needed to advance in the selection and design of more effective compounds with improved permeability but also in the optimization and validation of new screening tools for a more rapid and efficient identification of QS inhibitors.

7. References

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